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RADIATION BIOMARKER RESEARCH USING MASS SPECTROMETRY

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14. ABSTRACT Using mass spectrometry to investigate biomarkers from radiation exposure is fairly new. The implementation of mass spectrometric techniques to systems of biological interest has only recently taken off. Therefore, there have only been a limited number of applications of mass spectrometry in radiation dosimetry research. This review is intended to give an overview of mass spectrometry and its application to biological systems and biomarker discovery and how that might relate to relevant radiation dosimetry studies and how these two areas might be combined to benefit both areas of research. Efficient proteomics analyses have necessitated the use of various methods of mass spectrometry in recent years; especially concerning biomarker detection and characterization. A brief summary of the research thus far conducted follows.

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Abstract

Over 250 references are listed on SciFinder Scholar when searched for radiation biomarkers.[1] Less than 5% of those articles deal with mass spectrometry. This indicates an important area of research that still needs to be explored

Using mass spectrometry to investigate biomarkers from radiation exposure is fairly new. The implementation of mass spectrometric techniques to systems of biological interest has only recently taken off. Therefore, there have only been a limited number of applications of mass spectrometry in radiation dosimetry research. This review is intended to give an overview of mass spectrometry and its application to biological systems and biomarker discovery and how that might relate to relevant radiation dosimetry studies and how these two areas might be combined to benefit both areas of research.

Efficient proteomics analyses have necessitated the use of various methods of mass spectrometry in recent years; especially concerning biomarker detection and characterization. A brief summary of the research thus far conducted follows.

Figures

Introduction

Over 250 references are listed on SciFinder Scholar when searched for radiation biomarkers.[2] Less than 5% of those articles deal with mass spectrometry. This indicates an important area of research that still needs to be explored

Ruedi Abersold & Matthias Mann wrote an excellent primer[3] on the subject of proteomics in general. This paper covered each major method's advantages, basic instrumentation theory, and also a guide to selection of the proper method for the beginner. Careri and Mangia[4] identified the need for proper methods for maintaining precision. They proposed a standardization of practices to include consideration of all possible errors, since so many analytical methods are possible, each with their own strengths and weaknesses. Devoting their efforts to instrumentation, Fortier et al[5] designed a new microfluidic device to greatly enhance proteomic analyses. This apparatus significantly reduces postcolumn peak broadening and eliminates unnecessary deadspace volumes when performing liquid chromatography.

Following the experiment, the data must be analyzed. Frewen et al[6] proposed the use of spectral databases to characterize the results of MS/MS peptide spectra with excellent identification and sequencing accuracy. Their technique involved using a dot product similarity analysis to known compounds in existing databases. Since the field of proteomics is large indeed, Barbara Marte[7] contemplated the many aspects in a short overview of the subject. This essay informs the reader of difficulties in the field, and also some new advances. Reyzer and Caprioli[8] reviewed the present trends of MALDI analysis of tissue sections and wrote a general guide for sample preparation and analysis. Imaging of protein content is also covered in this work.

Further refining analytical resources, Rudnick et al[9] thoroughly analyzed the behavior of the MASCOT search algorithm in an effort to boost characterization accuracy. Their efforts greatly reduced false positive and false negative identifications. Bernd Simoneit[10] wrote a thorough review of current MS applications for biomarker detection in the environmental / geological field. Microbial, plant, and other biomarkers are discussed within, along with the most effective techniques for analyses. Combining methods can be most effective, as Taylor[11] discovered using LC/ESI/MS allowing them to identify very complex peptides and also biomarkers. This approach also affords the ability to quantize simultaneously while identifying peptides. Tu et al[12] used immobilized pH gradient / isoelectric focus (IPG-IEF) technique followed by electrospray-linear ion trap MS/MS. This approach, upon trypsin digest of the proteins contained on the gel sections, gave them the ability to detect more than 700 human proteins on a gel with only 300 µg of sample.

Mike Tyers & Matthias Mann[13] (also of the Abersold paper[1]) wrote an in-depth analysis of the progress of proteomics' various facets, and proposed necessary

advances for the field to mature. The existence of large data resources, free distribution of results, and cooperation of international efforts are all necessary events to achieve this.

Vitzthum et al[14] investigated the development of in-vitro diagnostics, and established guidelines for efficient design and implementation. These guidelines maintain that the IVD (in-vitro diagnostic) should be reliable, accurate, and highly selective to achieve optimum results. Vlahou and Fountoulakis[15] wrote a review of many different approaches in the search for disease biomarkers using electrophoresis and MS techniques. Wang et al[16] discovered that the comparison of proteomic datasets afforded discovery of metastatic cancer marking proteins using their novel algorithms.

Implementation

Alaiya et al[17] investigated novel methods of detecting tumor-derived proteins. This comprehensive paper also warns of common pitfalls of biomarker analyses. Gerd Assmann[18] chose HDL (high density lipoprotein) detection. Known as the "good cholesterol," its known anticoronary episodic properties can provide an excellent diagnostic tool to assess coronary health. Dalle-Donne et al[19] investigated in-depth the phenomenon of oxidative/nitrosative stress and its effect on proteins. They found that such altered proteins serve as efficient biomarkers for Alzheimer's, Lou Gehrig's, asthma, and others. Dong et al[20] created a GCMS method for analyzing acetone in blood; an indicator of diabetes. Their novel method consists of reaction of the acetone with a derivatization agent followed by GC/MS detection. This method allows for rapid determination of acetone levels without a complicated extraction process from the blood sample.

Ferdinandy and Schulz[21] reviewed the significance of nitric oxide, superoxide and peroxynitrite concentrations in the body. Excess peroxynitrite can cause myocardial ischaemia (restriction of blood flow to areas of the heart causing tissue death). This may serve as an excellent biomarker in the future to predict this deadly but common disorder.

Hewavitharana et al[22] encountered target compound loss subsequent to sample protein removal. A new, similarly structured internal standard was used, but loss still occurred. This was deemed to be caused most likely by the standards themselves binding to the proteins before removal. Hoos et al[23] created a new method for quantitative analysis of bioproteins in solution using immunoaffinity chromatography, protein digestion, and finally LC/MS. This approach is best suited for determinations of proteins in their biological contexts; in this work, the authors give diluted human plasma as an example of a typical context. Hoos et al were successful in automating this process as well.

Im et al[24] analyzed formaldehyde exposure effects in rats using MALDI/TOF, and also verified 7 proteins that could be definitive human biomarkers for excessive

formaldehyde exposure. This project's successful results can be applied to monitoring formaldehyde exposure in humans, leading to "sick building syndrome [22]". Ma et al[25] investigated a new technique for breath analysis of VOC content using MESI (membrane extraction with sorbent interface) that could be used to monitor acetone levels in diabetic patients via a non-invasive approach. MESI was chosen for its inherent selectivity and stability over current methods.

Neyraud[26] analyzed both whole and parotid saliva composition following stimulation of the subjects with different tastes (sweet, bland, sour, acid) using trypsin digests and MALDI analyses. They discovered that two known proteins, calgranulin A and Annexin A1 were nearly absent in parotid saliva, indicating that they most likely originate from other oral tissues.

Suriano et al[27] used SELDI/TOF analyses of thyroid biopsies to identify potential biomarkers for thyroid cancer. Since malignant as well as benign tumors respond similarly subsequent to staining, this project aims to detect more efficient biomarkers to afford accurate diagnoses. Wagner et al[28] used constant-neutral loss on a linear ion trap to discover biomarkers in human urine. This approach was tested using acetaminophen administered to test subjects, and the resulting marker (mercapturic acid) was identified with high precision, allowing quantitative analysis of the marker. Yan et al[29] studied the detection of N-terminally acetylated thymosin β_4, a suspected cancer biomarker, using ion trap tandem MS. This method yielded the ability to detect thymosin β_4 at the whole protein stage rather than at post-peptide digest.

Dosimetry studies

Brooks City-Base (formerly Air Force Base) has been the site of many dosimetry studies of varying subjects. Patrick Mason's team conducted many projects in this area; the first of which investigated the effects of 94 GHz radiation exposure on human volunteers.[30] This study discovered a significant relationship between cutaneous blood flow and skin heating rates upon irradiation. They[31] also determined that 94 GHz radiation did not have any appreciable effect on animal papilloma development. A later paper[30] investigated this subject further by using high and low power emissions: the findings were similar in that skin blood flow changes may greatly alter rates of heating upon exposure. In the next project[32], high-power pulsed microwave radiation was used on microbes, and the effects were noted similar to short-duration high temperature events. Mason and coworkers[33] examined millimeter wavelength radiation (30 to 300 GHz) and its potential for accidental overexposure; including short and long term effects. A review paper[34] was also published on such radiofrequency fields and their possible carcinogenic, mutagenic, and genotoxic effects. This project found, however, that according to the majority of papers published RF field exposure is not necessarily carcinogenetic, as is commonly alleged by popular myth.

William Hurt's group[35] also focused on RF exposure at 220 MHz. The main thrust of this experiment was to provide a complete mapping of the exposure set-up and to provide accurate dosimetry of the volunteers. The next project[36] involved 100 MHz RF with similar determination of accurate dosimetry. J. M. Muderwha et al[37] researched development of a multiparametric radiation biodosimetry system for applications ranging from medical personnel to spaceflight. Muderwha's colleague on that project, P. G. Prasanna, also worked on developing a computer application to determine total dose following radiation accidents.[38] This software package would allow management of the massive amounts of casualty data, including dosimetry and location at the time of incident to trace radiation patterns.

Mass Spectrometry of Radiation Biomarkers

Ménard and coworkers[39] have recently investigated the applicability of discovering clinical biomarkers of ionizing radiation using serum proteomic analysis. They used surface enhanced laser desorption ionization (SELDI) coupled with a high resolution time-of-flight (TOF) mass spectrometer to analyze 68 patient samples. The patients had a wide variety of diagnosis. This resulted in a wide variety of radiation exposures. Their results demonstrated the ability of the technique to distinguish between high and low dose radiation exposure. Computer modeling of the data enabled them to distinguish exposed from unexposed patient samples with 91% to 100% sensitivity. Their comment underscores our view that "Proteomic analysis for the discovery of clinical biomarkers of radiation exposure warrants further study".[39]

Ravant's group at DFRMC/SCIB in Grenoble, France have used mass spectrometry to investigate a variety of radiation induced biomarkers.[40-43] They used HPLC coupled with tandem mass spectrometry to investigate the radiation-induced base damage to isolated and cellular DNA.[40] A variety of bases were identified as resulting from radiation damage. The simultaneous determination of several base degradation products enables patterns to be discerned from the lesions. This can provide valuable mechanistic information regarding the formation of these radiation produced products. Cadet et al found that using HPLC and MS/MS in the multiple reaction monitoring mode was a sensitive method for singling our individual oxidized nucleosides.[41] They did a follow-on study examining aerated aqueous solutions of DNA that were exposed to ionizing radiation. The DNA was subsequently digested to nucleosides with a combination of endo- and exo nucleases. Four new lesions were found to be significantly generated.

Radiation Biomarkers at UTSA

The mass spectrometry group at the University of Texas at San Antonio analyzed a number of samples. The quality of some of the samples was suspect because of the poor quality of the data. All were mouse plasma. Seven of the samples

were microwave exposed mice, the other seven were sham. The samples were prepared by acid precipitation of abundant proteins (0.1% acetic acid). They were then spun through a 10KDa cutoff filter in order to isolate the peptides. The samples were then desalted by washing them over a C18 spin column using 0.05% TFA as an ion-pairing reagent. The samples were then spotted onto a 200µ Anchorchip with 1:1 saturated HCCA matrix in ethanol. The samples were then analyzed by MALDI-TOF (750-9000 m/z, 300 shots per spot avg.) The spectra were extremely noisy and complex. The data was of insufficient quality to obtain definitive biomarkers.

Trips were also made to AFRL/HEDR at Brooks City Base to assist with their sample analysis using the Finnigan LTQ located there. Mr. Mullens and Ms. Nagore assisted with training personnel at AFRL/HEDR and when necessary with the operation of the LTQ. Five saliva samples from AFRL/HEDR were also analyzed at UTSA. The results are presented below. No background was given for these samples so just the raw results are presented for further interpretation.

Saliva Proteome Research

Introduction

The salivary proteome, the collection of proteins found in saliva, is a rich source of information that can be used to clinically assay health and physiological status. Specifically, samples of blood, saliva, hair or other readily sampled tissues, obtained from different human populations can be compared to identify chemical components associated with a particular disease or change in physiological state. Components identified through comparative analysis of composition are termed biomarkers.

A major impediment to the identification of biomarkers is their low abundance compared to other components of similar chemical composition, i.e. proteins and peptides. For example, in plasma, 2 proteins (albumin and IgG) make up approximately 70% of the total weight of all proteins present.[44] In saliva, and other tissues, a small number of proteins tend to dominate as well. These common, non-biomarker proteins, complicate analysis by causing reduction of sensitivity (peak suppression) in direct mass spectrometry based methods including MALDI/MS and distortion of migration and low abundance spot suppression during 2-dimensional gel electrophoresis. There are several remedies that can reduce or eliminate the impact of common proteins and peptides on biomarker discoveries. First, a sample can be passed through a column to which specific antibodies directed against the most common proteins have been attached to the stationary phase.[44] For plasma, these columns have proven effective at improving the detection sensitivity for proteins and peptides present in low abundance and thus increasing the number of biomarkers found during discovery. For saliva, these columns do not yet exist.

Another remedy is to bind chemically distinct subsets of the biological sample to a stationary phase. For example, relatively lipophilic proteins and peptides may be bound to a stationary C-18 or C-8 phase whereas other proteins, including highly abundant hydrophilic proteins and peptides, remain in mobile phase. In practice this can be accomplished by using magnetic beads coated with a defined chemical matrix to capture specific chemical classes, eluting with the captured components with a buffer. Because the complexity of the sample is relatively low, the eluted peptides and proteins are ideally suited for MALDI/MS based biomarker discovery. MALDI/MS methods have several advantages over other methods and include high-throughput capability and ease of automation.

There is a growing interest in using a saliva matrix for clinical diagnostic testing. This is due to the non-invasive sample collection and lower cost of collection and storage compared to serum. Saliva represents an important source of diagnostic biomarkers. Its composition correlates with many proteins found in serum that have been shown to be informative in determining disease susceptibility and progression. For instance gamma-glutamyl-transferase (GGT) and β2-microglobulin (β2m) were both quantified in a study of Sjögren syndrome by Jimenez-Alonso et al.[45] Using an enzyme immunoassay both GGT and β2m could be detected in saliva and serum. Another marker that has been studied in saliva and serum is estradiol. Tivis et al[46] collected samples from postmenopausal women to decide if saliva could be used instead of serum in quantitative analysis using estradiol radioimmunoassay kits. Estradiol has been found to correlate with endocrine function, coronary artery disease, and breast cancer. It was concluded that estradiol could only be accurately detected in women that underwent estrogen therapy otherwise estradiol levels were too low. There are however biomarkers that do not have these restrictions such as cortisol. Cortisol is known to increase in concentration during exercise. In past years cortisol has been measured in serum and saliva using radioimmunoassay. Gozansky et al[47] showed that by using an enzyme immunoassay, serum and saliva had cortisol at detectable limits and gave complementary results.

Saliva is secreted in the mouth from three major glands (parotid, submandibular, and subliqual) and is composed of water, electrolytes, proteins, enzymes, and mucins.[48] A major fraction of the salivary proteome is proline rich proteins (PRP). Basic PRPs are known to be secreted mainly from the parotid gland whereas acidic PRP along with lysozymes, histatins, and statherin are secreted from the submandibular and parotid glands.[49] Cystatins are only secreted from the submandibular gland. Mucins, along with other salivary proteins, aggregate into high molecular weight glycoproteins making saliva difficult to analyze directly using mass spectrometry.[50]

Currently, the saliva proteome is predominately studied using two-dimensional gel electrophoresis as a separation technique and mass spectrometry to identify proteins. However, there are many limitations to this method including the inability to identify large or small molecular weight proteins, as well as, highly

acidic, highly basic, or hydrophobic proteins from the same sample.[51] There are also limits in detection due to staining methods that only have a dynamic range of about three to four order of magnitude in mass and concentration. Owing to these shortfalls in current two-dimensional gel technology, we began to investigate other methods for salivary biomarker identification. Using magnetic bead sample preparation techniques with saliva samples and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS), we have been able to reproducibly generate mass spectrometric protein profiles that can be used for biomarker discovery.[52, 53] Not only are the magnetic beads used to reduce the sample complexity, they also remove many of the salts, buffers, and detergents that hinder mass spectrometric analysis. The magnetic beads also aide in concentrating the peptide/proteins of interest.

Experimental Procedures

Equipment

Mass spectrometric data was obtained by using a Reflex III MALDI-TOF (Bruker Daltonics, Billerica, MA). MALDI-TOF spectra were acquired in linear mode with a pulsed nitrogen laser (337nm) with a pulse rate of 10 Hz. Ion source 1 was set to 20 kV, Ion source 2 was set to 18.5 kV and the lens was set to 7 kV. 1000 scans were averaged for each sample to improve spectral quality. Peptide/protein peaks were measured in three different sets, 1000-3000 Da, 3000-10,000 Da, and 5000-20,000 Da. A fixed laser power of 65% was used with all mass ranges. Instrument control was carried out by using FlexControl software (version 1.0, Bruker Daltonics, Billerica, MA) and data processing was carried out using FlexAnalysis (version 2.2, Bruker Daltonics, Billerica, MA).

Clinical saliva samples - Basic collection method

Saliva is collected using a Saliva Sampler (Saliva Diagnostic Systems, Inc., Medford, NY) which consists of an absorbent pad and a preservative transport buffer solution. The absorbent pad was placed under the tongue until the pad was saturated and the white indicator turned blue. The pad is placed in the collection tube with buffer and the sample is extracted through a porous plastic filter. Saliva was immediately mixed with protease inhibitor that was supplied with the absorbent pad to preserve the sample protein composition. This is necessary in order to have a protein solution compatible with available data analysis software and also have accurate comparisons between samples. The samples are aliquoted into tubes and stored at -80 ºC.

Clinical saliva samples - Alternative collection method

Alternatively, unstimulated saliva is collected directly into 50 mL conical centrifuge tubes on ice and vortexed briefly. 1 mL of the sample is combined with

0.5 μL of 10X protease inhibitor cocktail (Sigma, cat. # P2714), vortexed for 1 minute, and stored at -80 ºC. After thawing, 500 μL of treated sample is transferred to a fresh microcentrifuge tube containing 500 μL of 0.23% TFA (Aldrich, 99% purity, cat. # 10,623-2) and vortexed again for 1 minute. Following centrifugation at 13.5 KRPM for 10 minutes, 800 μL of supernate is transferred to a fresh tube, used for further analysis, or stored at -80 ºC.

Saliva sample preparation - Magnetic bead method

To enrich for proteins and peptides and to remove buffers and salts, a C-18 (MB-C18, 223325) magnetic bead profiling kit from Bruker Daltonics, Billerica, MA is used. 15 µL of precleared saliva is mixed with 10 µL magnetic beads and 10 µL binding solution in a standard thin wall PCR tube and incubated for five minutes at room temperature. Tubes are placed in a magnetic bead separator (Bruker, 65602) and supernates are pipetted out. Tubes are removed from the magnetic bead separator and 100 µL of the Bruker washing solution is added to the magnetic beads, mixed thoroughly, and placed back into the magnetic bead separator. Again, supernates are pipetted out and disposed of to ensure that all salts, buffers, and detergents are removed from the sample. The washing procedure is repeated twice. Finally, 5µL of the Bruker elution solution is added to the beads and mixed. The PCR tube is placed back into the magnetic bead separator for two minutes. Eluates are pipetted out into a clean tube and mixed with 5 µL stabilizing solution. The solution is then mixed with cinnamic acid (Sigma, C-2020) and spotted on an Anchor Chip© (Bruker Daltonics). A control spot is also added that contains a 1 µM solution of cytochrome C as an internal standard for MALDI-TOF analysis.

Saliva sample preparation - Direct analysis method

Fresh matrix solution is prepared with 50 mg cinnamic acid (Sigma, cat. # C-2020), 1 mL solvent containing 1 part absolute ethanol and 2 parts acetone. 1 μL of precleared saliva is mixed with 1 μL matrix solution in a 100 μL PCR tube and spotted onto an Anchor Chip. After adding a cytochrome C control spot, the chip is dried for a minimum of 1 minute under vacuum prior to MALDI-TOF analysis.

Results

Five saliva samples were collected at AFRL/HEDR from anonymous donors without retaining any donor information (Patrick Mason, PI, "Exempt" protocol #F-BR-2006-0006-E, approved by Col. Calcote, IRB Chair, Brooks City-Base, 06 Oct 2005), following the alternative method. These samples were prepared for direct MALDI-TOF analysis without C18 fractionation. Three mass spectra were obtained for each sample, which are presented in Figures 1 to 15. One spectrum is of the low mass range between 1,000 and 3000 Daltons, Figures 1 to 5. The second one is of the mid range between 2,000 and 10,000 Daltons, Figures 6 to

10. The third is of the high mass range between 5,000 and 20,000 Daltons, Figures 11 to 15. Table 1 list observed peaks in these spectra for which assignments were possible using literature sources.[50, 54]

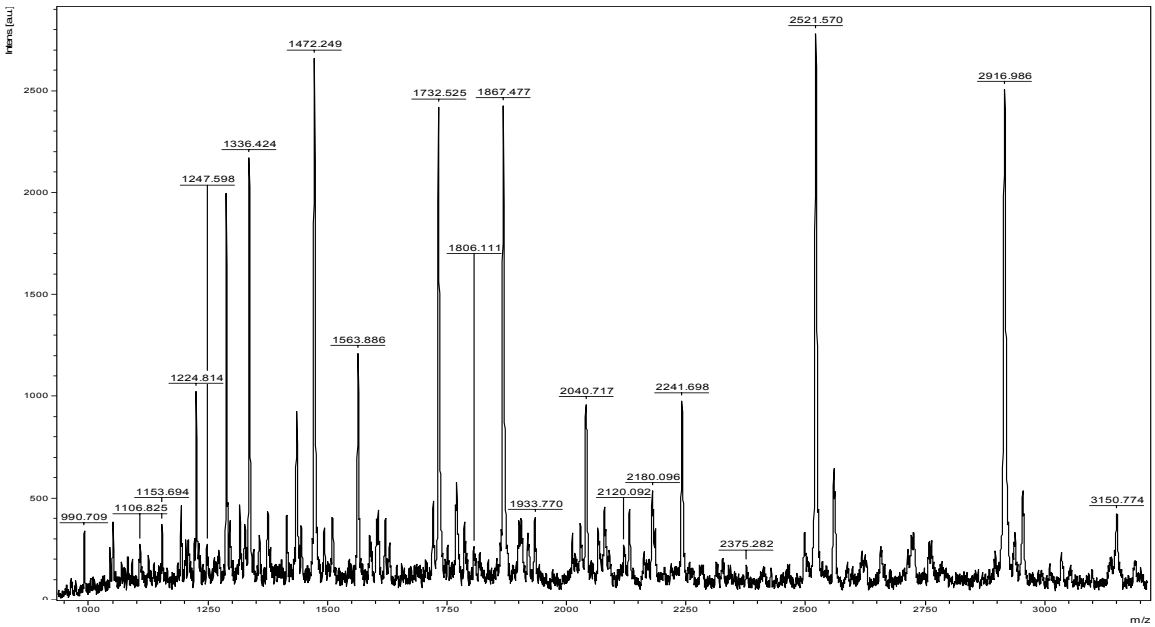

Figure 1. Saliva sample 1. Low mass range MALDI-TOF m/z 1000 to 3000

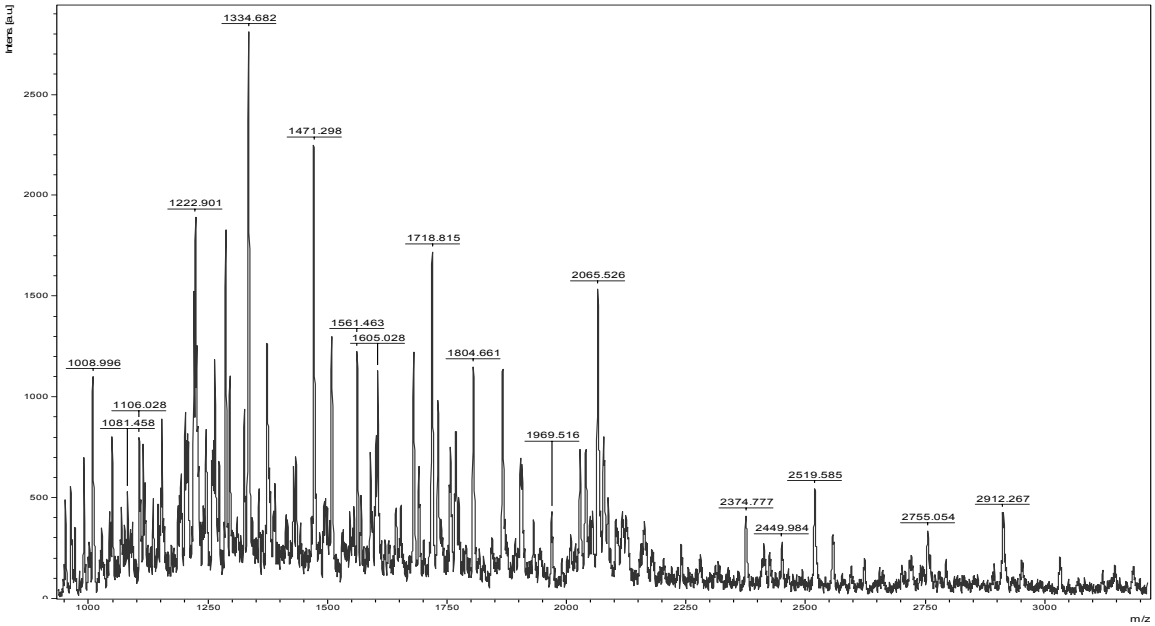

Figure 2. Saliva sample 2. Low mass range MALDI-TOF m/z 1000 to 3000

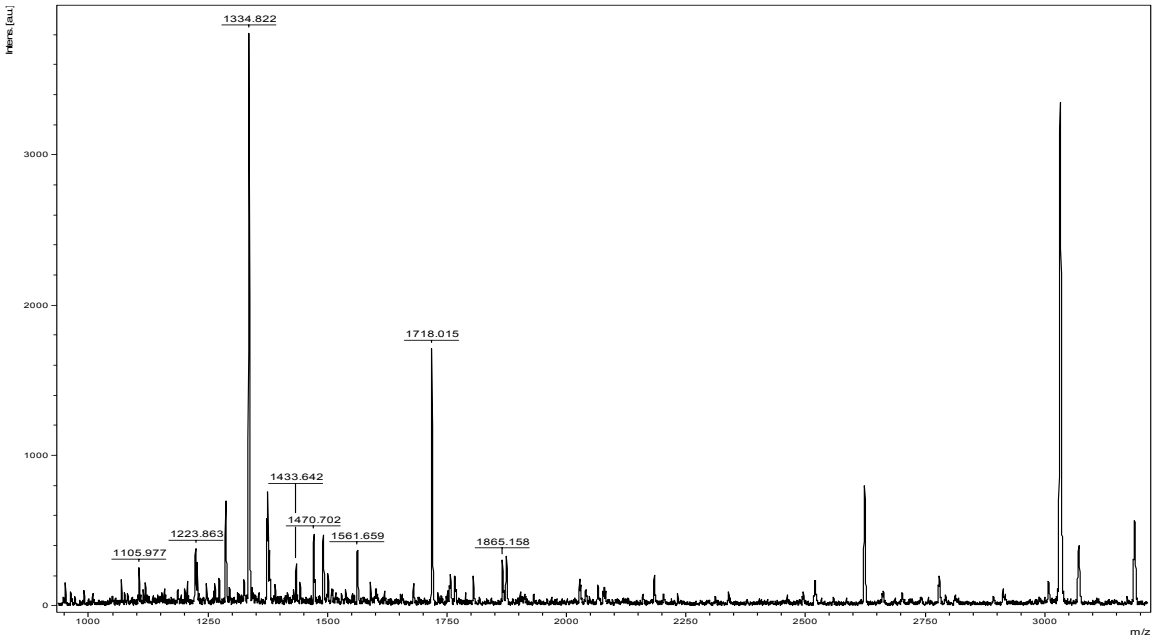

Figure 3. Saliva sample 3. Low mass range MALDI-TOF m/z 1000 to 3000

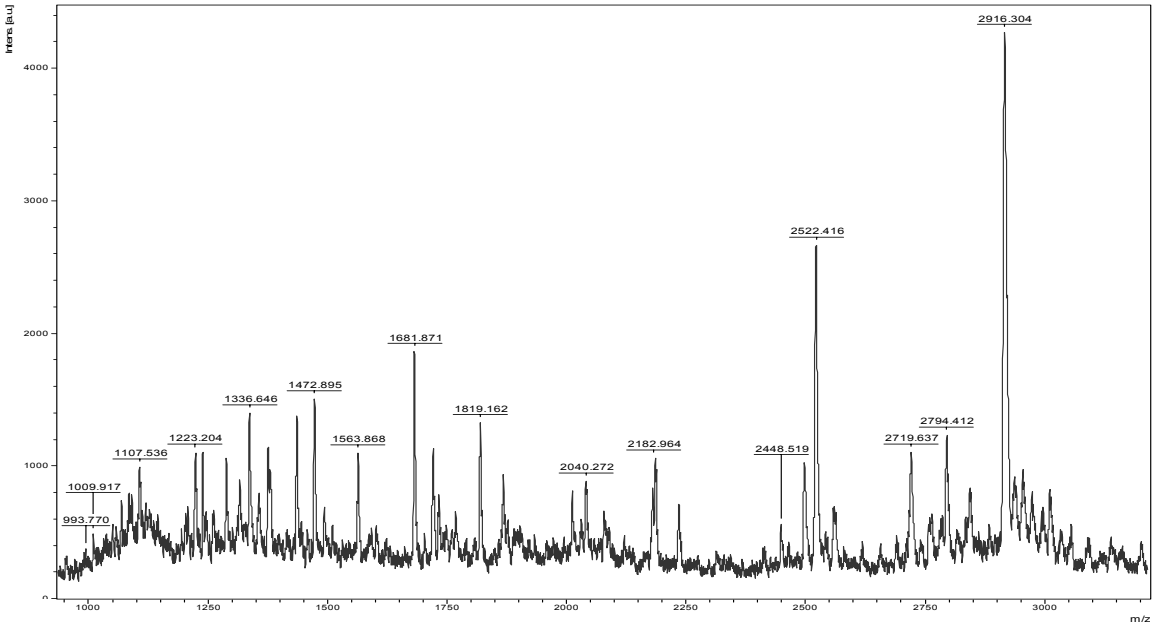

Figure 4. Saliva sample 4. Low mass range MALDI-TOF m/z 1000 to 3000

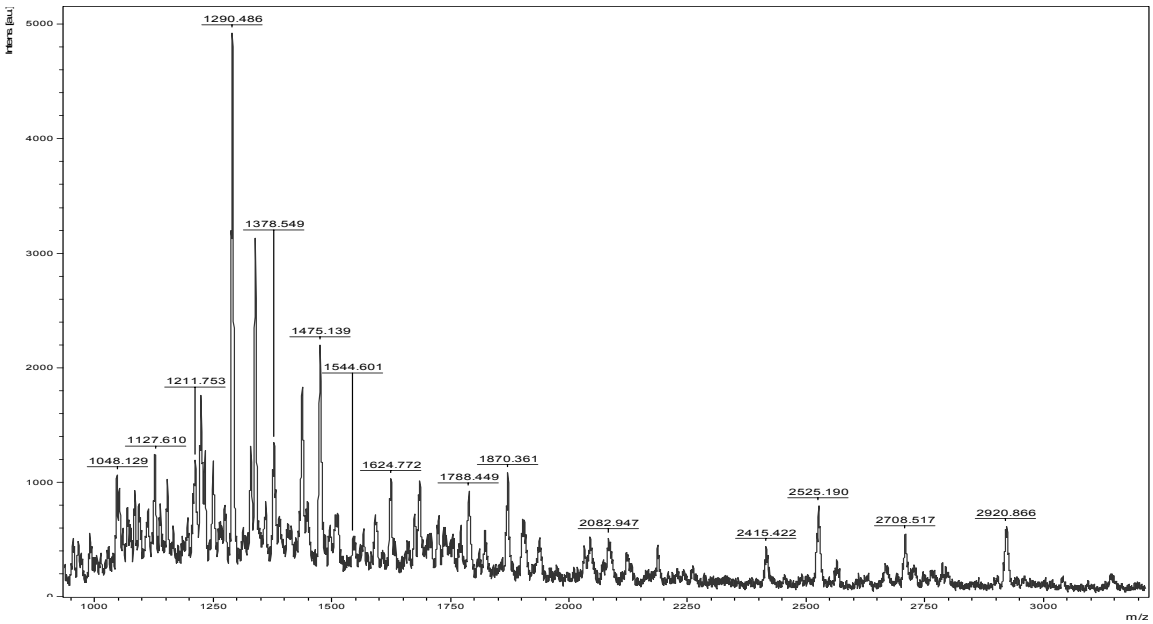

Figure 5. Saliva sample 5. Low mass range MALDI-TOF m/z 1000 to 3000

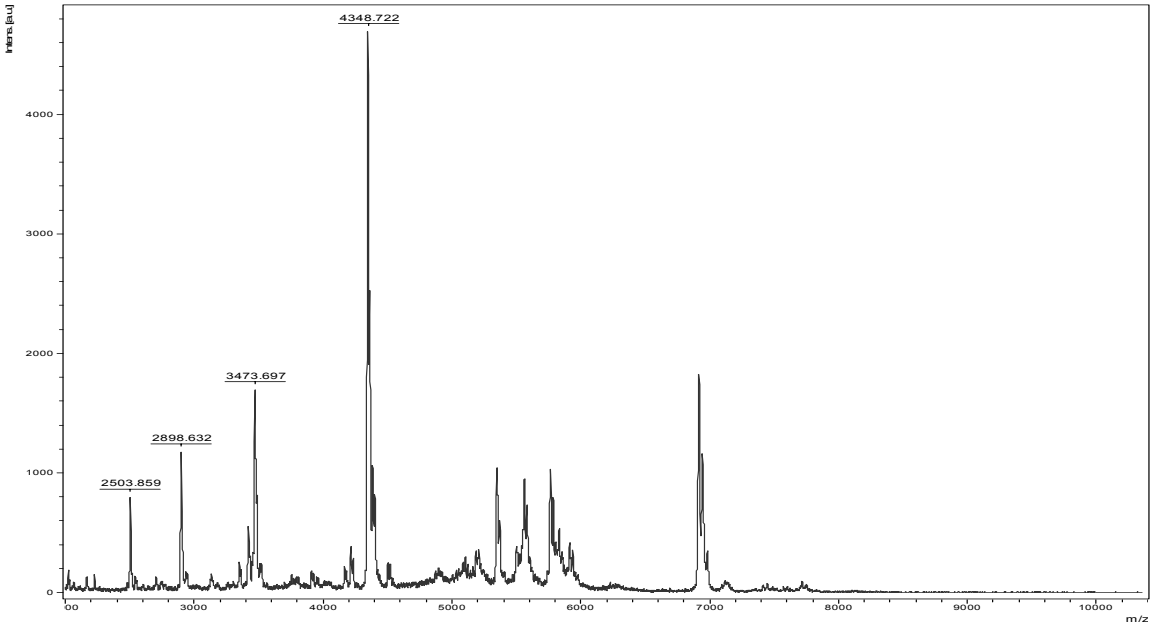

Figure 6. Saliva sample 1. Mid mass range MALDI-TOF m/z 2000 to 10,000

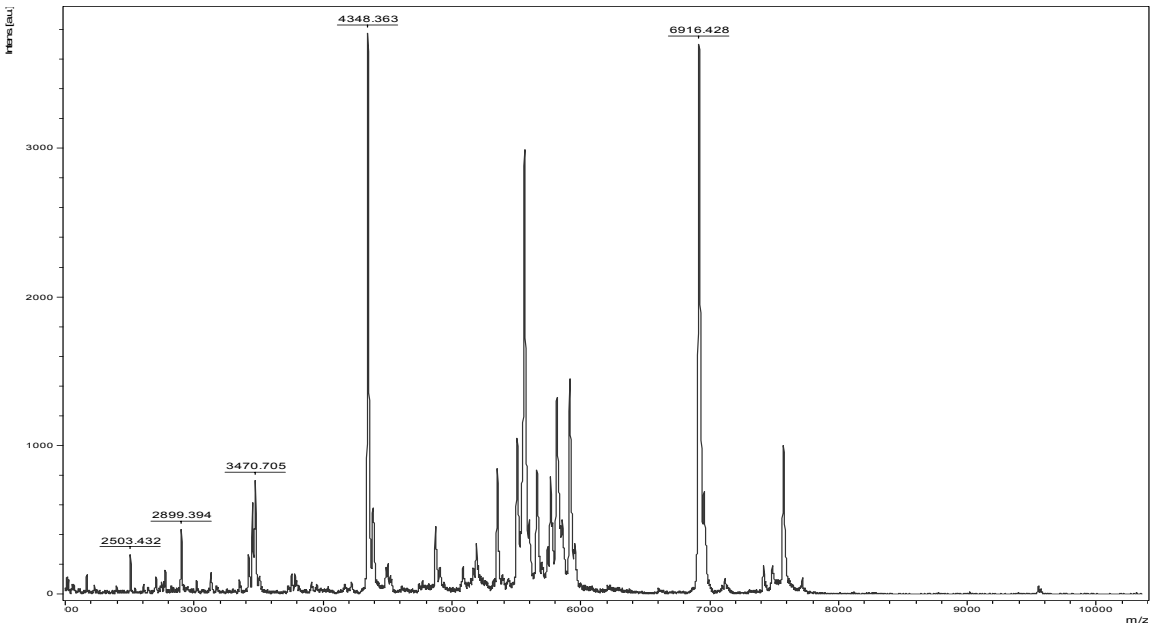

Figure 7. Saliva sample 2. Mid mass range MALDI-TOF m/z 2000 to 10,000

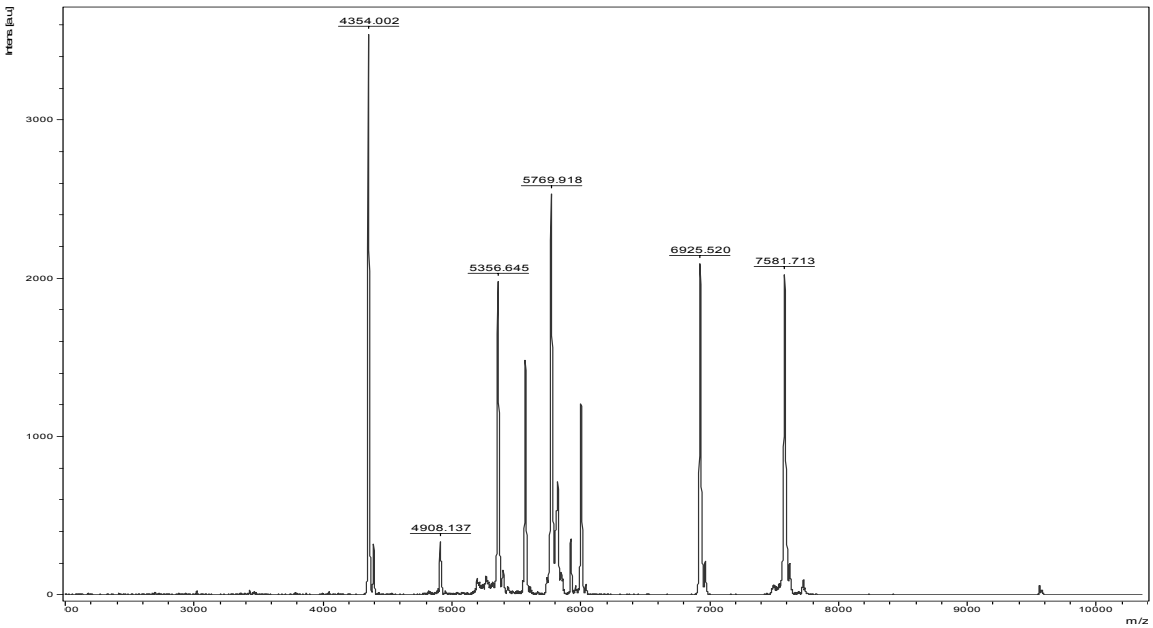

Figure 8. Saliva sample 3. Mid mass range MALDI-TOF m/z 2000 to 10,000

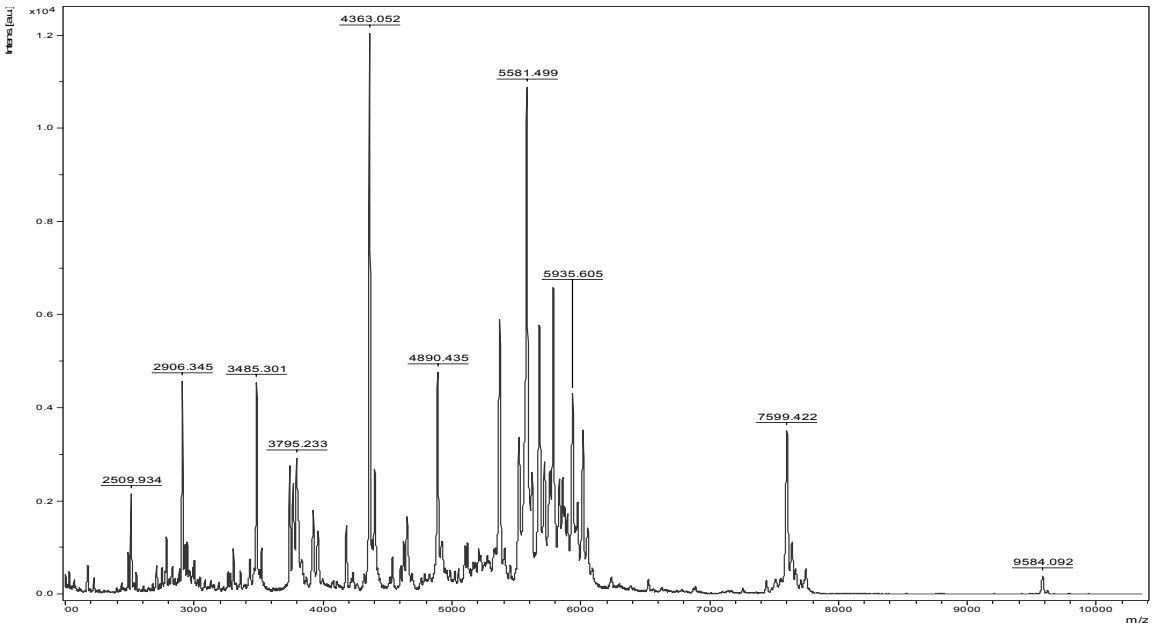

Figure 9. Saliva sample 4. Mid mass range MALDI-TOF m/z 2000 to 10,000

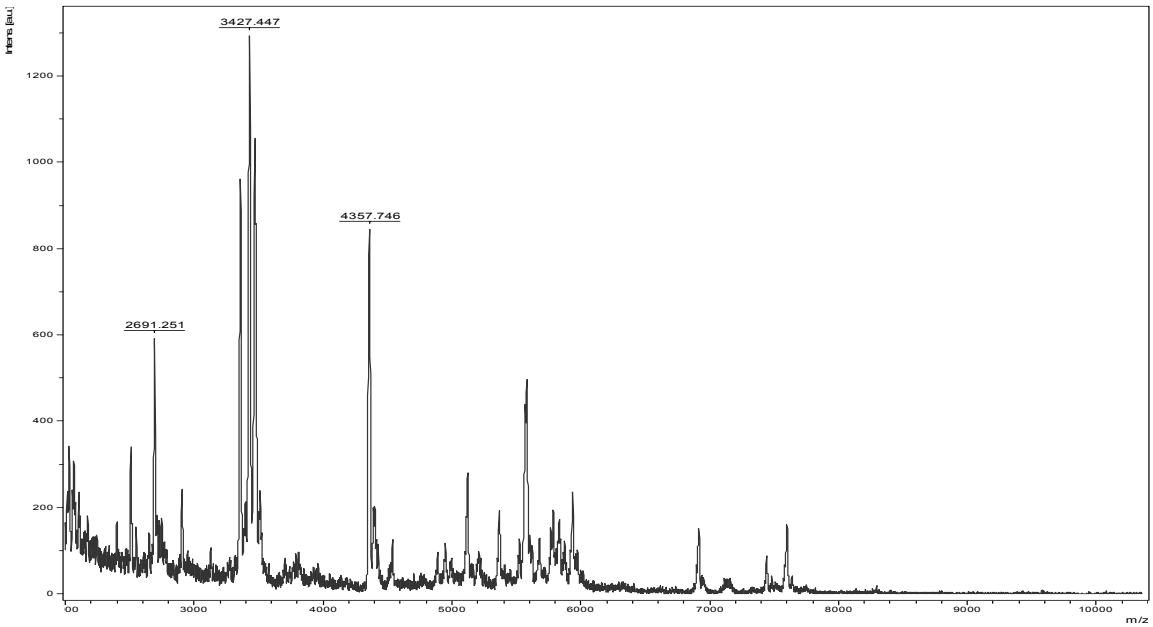

Figure 10. Saliva sample 5. Mid mass range MALDI-TOF m/z 2000 to 10,000

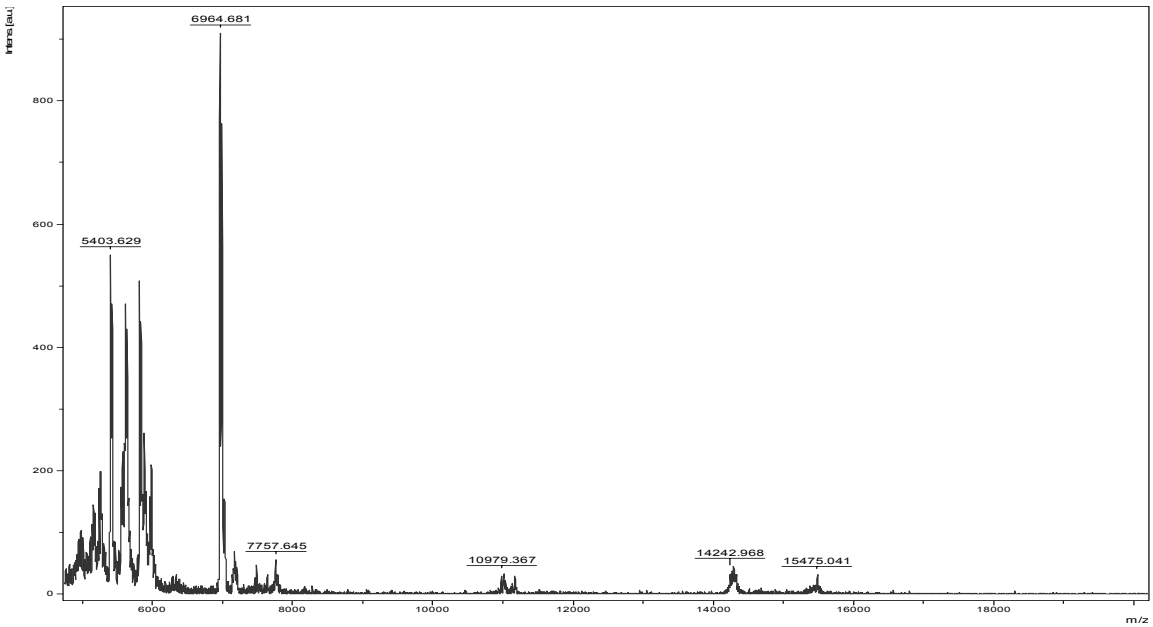

Figure 11. Saliva sample 1. High mass range MALDI TOF m/z 5000 to 50,000

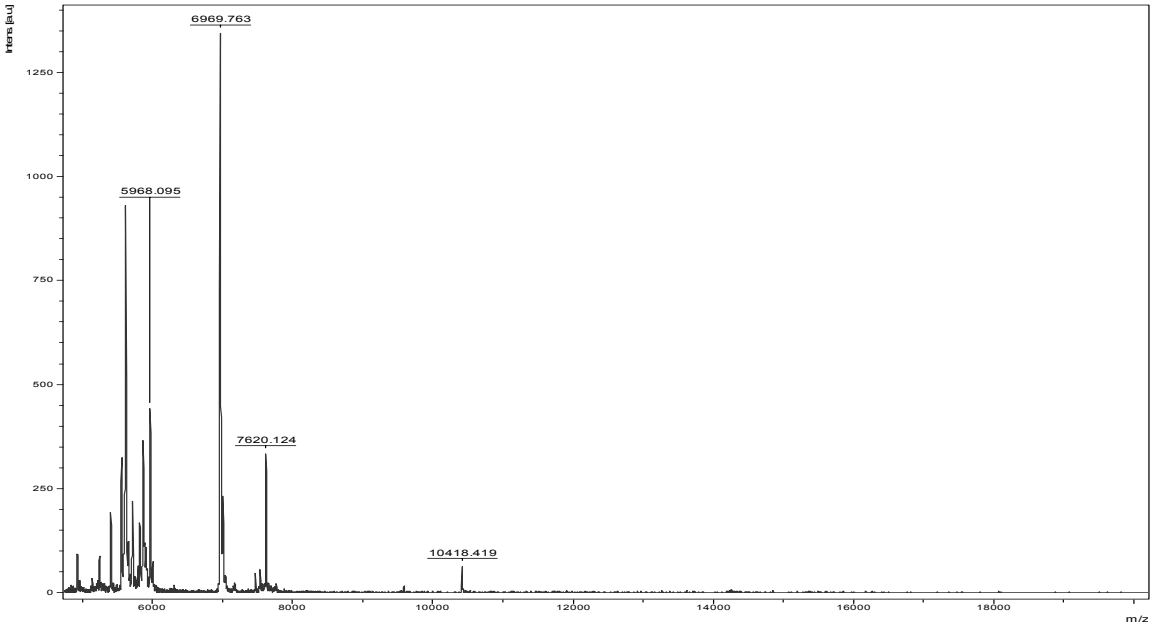

Figure 12. Saliva sample 2. High mass range MALDI TOF m/z 5000 to 50,000

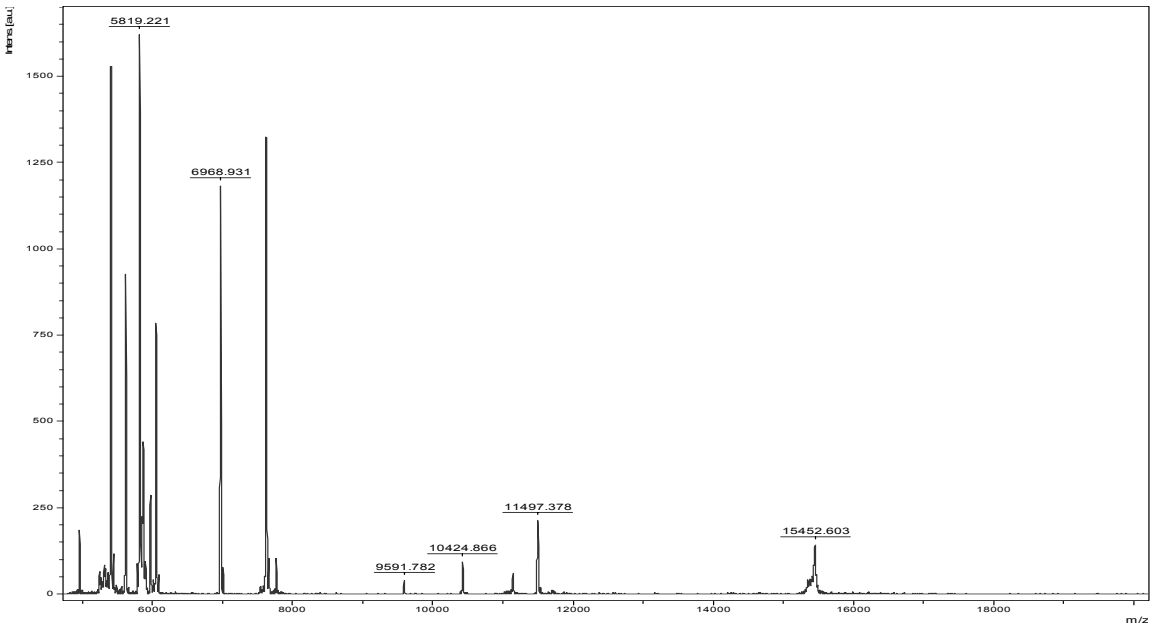

Figure 13. Saliva sample 3. High mass range MALDI TOF m/z 5000 to 50,000

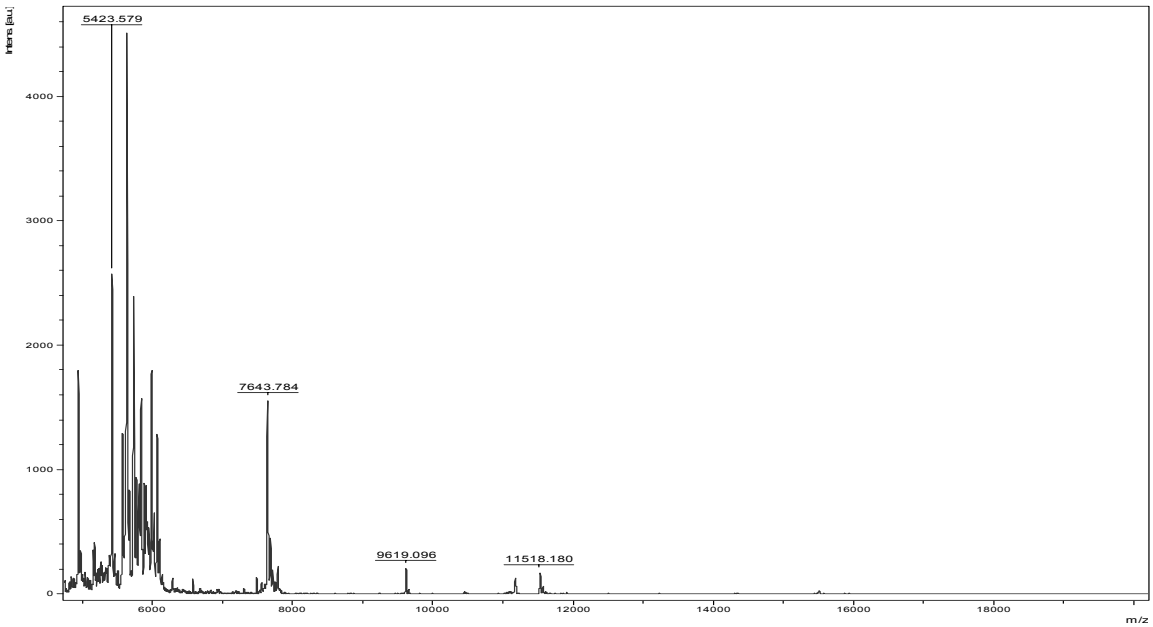

Figure 14. Saliva sample 4. High mass range MALDI TOF m/z 5000 to 50,000

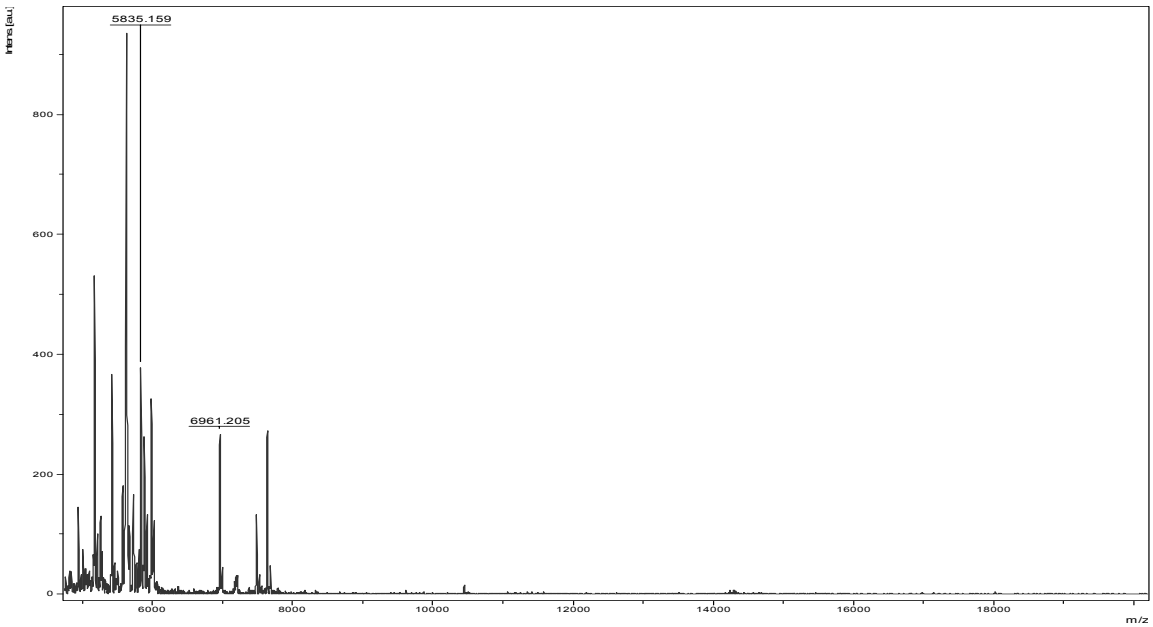

Figure 15. Saliva sample 5. High mass range MALDI TOF m/z 5000 to 50,000

Table 1: Peak assignments for mass spectra.

Experimental m/z	Theoretical m/z	Observed in Saliva sample	Area	Possible Peptide [1]
1081.5	1081.6	low	494	Histatin 11
1286.8	1287.6	low	3730	Histatin 3
1434.4	1434.7	low	1225	D1A
1561.5	1562.8	low	2870	Histatin 8
1680.0	1680.9	low	3449	Histatin 7
1718.8	1718.9	low	5123	Histatin 10
2917.0 2906.9	2911	low, mid	39632	T-cell receptor delta chain [2]
3484.0	3492.6 3490	mid	48723	Defensin 3 Defensin HNP-3, Chain A [2]
4361.5	4369.2	mid	149619	IB8(P-C)
5264.0	5263.4	high	1087	Statherin isoform
5390.6	5378.5	high	1424	Statherin
5580.3	5587.8	mid	171898	IB4(P-H)
5760.8, 5771.5	5767.9	mid, high	7165	IB7
5785.8	5790.0	mid	51444	PRP3 isoform
5834.1, 5839.6	5840.0	mid, high	3458	Basic P-F
6015.7, 6027.2	6021.9	mid, high	30122	IB9(P-E)
6140.7	6145	high	1182	Histatin 3 precursor [2]

Discussion

The spectra demonstrate the good points and the bad points when it comes to the analysis of saliva samples. Although there is variability in the observed species there is also some overlap. Many of the observed species have not been identified. Those for which literature references could be found are listed in Table 1. The variability in the spectra are due to a variety of sample collection factors which range from time of day, what had been eaten, and the general health of the individual. For these samples we had no control over how, when, and from whom the samples where collected. The data presented is intended to be a survey of the peptides and proteins present in the samples which is useful for a finger print comparison between samples.

The analysis of saliva as a diagnostic tool may one day be useful when the saliva proteome is better understood. At present are the issues of sample reproducibility between individuals and across populations. The analytical tools for the analysis of such complicated samples are just now reaching the point where they can grapple such a complicated task.

Future work should focus on obtaining sufficiently large sample sets so that the impact of individual sample variability will be minimized. It still needs to be determined what is in a ‘normal’ saliva sample before extrapolations can be made as to what is a useful and informative biomarker. Analysis of these datasets would be accomplished using software tool like the ClinPro software from Bruker. Once sufficient resources have been invested to resolve these issues through the generation of the necessary databases, the application of mass spectrometry to identify and characterize salivary biomarkers will become the method of choice.

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2. Search done in Feburary 2007
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